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Antibody-mediated opsonization of red blood cells in parvovirus B19 infection

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ABSTRACT

Red blood cells (RBCs) express abundantly parvovirus B19 receptor, and their role in the dissemination or clearance of B19 infection is unknown. In this study, we report that in early, acute or persistent infection, B19 viremia is mostly associated with RBCs. The capacity of different patients' plasma or IgG to opsonize RBCs collected from patients with early B19 infection, was investigated. The highest opsonization activity was observed with plasma or IgG fractions from patients with past B19 infection. In contrast, IgG samples from patients with acute or persistent infection showed no or little opsonization activity. The depletion of antibodies specific to B19 VP1, but not VP2, from IgG samples, resulted in a significant suppression of opsonization. Furthermore, IgG samples preincubated with heated B19 particles exposing VP1-unique (VP1u) region were unable to opsonize RBCs. These observations clearly suggest a role for anti-VP1u IgG in the opsonization of RBC-bound B19 particles.

Introduction

Human parvovirus B19 (B19) causes erythema infectiosum in children (Anderson et al., 1983), and is also associated with a wide spectrum of clinically symptoms, such as arthropathy (Reid et al., 1985), anemia (Serjeant et al., 1981), myocarditis (Porter et al., 1988) and hepatitis (Yoto et al., 1996). Parvovirus B19 capsid is composed of the structural proteins, VP1 (minor, 5%) and VP2 (major, 95%), which are identical except for the additional VP1-unique region (VP1u), at the amino-terminal end of the VP1-protein (Cotmore et al., 1986; Ozawa and Young, 1987). The most amino-terminal part of VP1u region harbors strong neutralizing epitopes (Kurtzman et al., 1989a; Anderson et al., 1995; Zuffi et al., 2001), whereas the carboxy-terminal end of VP1u harbors a phospholipase A_2 (PLA_2)-like motif (Dorsch et al., 2002), which is considered to play an important role in the induction of inflammatory processes (Lu et al., 2006). These two critical parts of VP1u have been shown to occupy an internal position in the virion capsid, but a conformational change renders these regions accessible (Ros et al., 2006).

Parvovirus B19 viremia has been shown to precede the appearance of B19-specific IgM (Patou et al., 1993, Cassinotti et al., 1993), and to be crucial for the early detection of B19 infection, especially in immunocompromised patients (Marchand et al., 1999; Moreux et al., 2002), and patients with hematological disorders (Mishra et al., 2005). Furthermore, B19 DNA load has been shown to correlate with the phase of infection (Manaresi et al., 2002), and its determination is thought to be useful for monitoring persistent B19 infection in high-risk group patients (Bergallo et al., 2006). Parvovirus B19 viremia has been always investigated in serum or plasma of patients with B19 infection. However, in a recent study, the analysis of a blood sample from one patient with recent B19 infection (IgM and IgG positive) has revealed that more than 90% of the virions circulating in the blood were associated to RBCs (Bönsch et al., 2008). Human mature red blood cells (RBCs) express high levels of B19 receptor, the P antigen (Fletcher et al., 1979), but are not permissive for parvovirus B19 entry (Weigel-Kelley et al., 2003). Binding of B19 particles to RBCs has been shown to expose the amino- and carboxy-terminal parts of VP1u (Bönsch et al., 2008). The role of RBCs in the dissemination or clearance of B19 infection is still unknown. In the current study, we provide evidence that B19 viremia is mostly associated with RBCs, and that RBC-bound B19 particles can be efficaciously opsonized in vitro by specific antibodies that are potentially directed against VP1u, prevalent in patients with past B19 infection, and uncommon in patients with persistent B19 infection.

Results

Parvovirus B19 infection in clinical samples

The characteristics of the study population are summarized in Table 1. The frequencies of B19 DNA and B19-specific IgM and IgG positivity in different plasma samples are given in Table 2. All patients with detectable B19 DNA in plasma had detectable B19 DNA in RBC fractions. Patients with B19 infection were grouped according to the stage of infection, which was inferred from a combination of clinical data and results of PCR and serology (Table 3). In either plasma or RBC

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Table 1
Baseline characteristics of the study population.

	Control subjects[a]	Patients with fever and rash[b]	Patients with chronic hemolytic anemia	Kidney transplant recipients with aplastic crisis
n	100	138	162	40
Gender				
M	63 (63%)	85 (62%)	96 (59%)	29 (72%)
F	37 (37%)	53 (38%)	66 (41%)	11 (8%)
Age (range)[c]				
Children	7 (4–15)	7 (4–12)	6 (4–10)	Null
Adults	40 (16–70)	40 (16–69)	45 (27–58)	52 (45–66)

[a] Control subjects included subjects tested negative for B19 DNA, IgM and IgG.
[b] Some patients with fever and rash have later developed arthralgia.
[c] Data are represented by the median of age in years.

fraction, the highest median viral load was observed in early B19 infection, whereas the lowest median viral load was observed in resolving infection (Fig. 1A). The median B19 viral load in chronic infection was significantly higher than that in resolving infection ($p<0.0001$), and significantly lower than that in acute infection ($p<0.0001$). Despite the fact that B19 viremia was mostly associated with RBCs (Fig. 1B), a low but significant correlation was obtained between plasma and RBC viral load ($r^2=0.33$, $p<0.001$, Fig. 1C).

Opsonization of RBCs isolated from patients with B19 infection

The capacity of different patients' plasma to opsonize RBC-bound B19 particles (10^4 copies/RBC) isolated from patients with early B19 infection, was determined using erythrophagocytosis assay. As shown in Fig. 2, the highest phagocytic index was observed when RBCs were opsonized with plasma from patients with past B19 infection. The opsonization activity of plasma in resolving B19 infection, was variable between patients, but was significantly higher than that in acute ($p<0.0001$) or chronic B19 infection ($p<0.0001$). Plasma from B19-seronegative control subjects and from patients with early B19 infection showed very low opsonization activity (Fig. 2).

Identification of plasma opsonic factor

IgG eluted from plasma of patients with past B19 infection, showed dose-dependent opsonization activity, whereas no significant opsonization activity was observed using IgG eluted from plasma of B19-seronegative subjects (Fig. 3A). To confirm that the observed opsonization was mediated through Fc-gamma receptors (FcγR), monocytes were preincubated at 37 °C for 30 min with 0.01 μg/μl of monoclonal IgG1 antibodies directed against FcγRI, FcγRII or FcγRIII. Following removal of unbound antibodies by three times washing with PBS, monocytes were used in the erythrophagocytosis assay. A significant impairment of erythrophagocytosis was observed with anti-FcγRII and anti-FcγRIII antibodies ($p<0.0001$, Fig. 3B). The inhibition of erythrophagocytosis was maximal when antibodies against FcγRII and FcγRIII were mixed together before being added to the monocytes. No significant inhibition of erythrophagocytosis was observed when monocytes were preincubated with anti-FcγRI antibodies or with the mouse IgG1 control.

Table 2
Markers of parvovirus B19 infection in clinical plasma samples.

Condition	*n*	B19-DNA positive-patients *n* (%)	B19-specific IgM positive-patients *n* (%)	B19-specific IgG positive-patients *n* (%)
Fever and rash	138	17 (12)	21 (15)	32 (23%)
Chronic hemolytic anemia	162	28 (17)	25 (15)	44 (27%)
Transient aplastic crisis following kidney transplantation	40	4 (10)	4 (10)	19 (47.5%)

Table 3
Number of patients grouped according to the stage of parvovirus B19 infection.

Stage of B19 infection	B19-specific symptoms[a]	B19-specific markers	*n*
Early infection (preseroconversion)	+ or −	DNA+, IgM−, IgG−	20
Acute infection	+	DNA+, IgM+, IgG+	15
Acute resolving infection	+	DNA−, IgM+, IgG+	35
Past infection	−	DNA−, IgM−, IgG+	31
Chronic infection	+	DNA+, IgM+ or−, IgG+	14

[a] B19-specific symptoms included erythema over the malar areas, maculopapular rash on the trunk and limbs, arthralgia, anemia-related symptoms or aplastic crisis.

Identification of the target of opsonizing antibodies

In order to ascertain that opsonizing IgG recognize B19 particles bound to the surface of RBCs, RBCs from patients with early B19 infection (10^4 B19 DNA copies/RBC), were treated with trypsin/EDTA and washed with PBS prior to the erythrophagocytosis assay; trypsin/EDTA treatment has been previously documented to remove completely the uninternalized B19 particles bound on the surface of RBCs (Weigel-Kelley et al., 2003). As shown in Fig. 4A, no significant opsonization activity was detected following treatment of RBCs with trypsin/EDTA. To confirm these findings, RBCs from control subjects were sensitized with 2×10^8 B19 copies/ml, and then used in the erythrophagocytosis assay. A high percentage of phagocytic activity was obtained in the presence of B19-sensitized RBCs, whereas no significant phagocytosis was observed in the presence of nonsensitized RBCs (Fig. 4B).

In order to identify the target for the plasma IgG that mediate opsonization of RBC-bound B19, IgG specific to B19 VP2 antigen were depleted from IgG samples isolated from patients with past B19 infection, prior to their use in the erythrophagocytosis assay. The results showed no significant impairment in the opsonization activity of IgG (Fig. 5A). The presence of anti-VP1 antibodies in IgG samples depleted of anti-VP2 antibodies was confirmed by western blot (Fig. 5B). However, when IgG specific to B19 VP1 were depleted from IgG samples, a significant inhibition of erythrophagocytosis was observed (Fig. 5A). Western blot results showed no reactivity of IgG depleted of anti-VP1 antibodies with VP1 protein (Fig. 5B). Unlike anti-VP1 antibodies, antibodies directed against denatured VP2 protein were not detected by western blot, even before performing depletion tests. Since the depletion of anti-VP1 antibodies but not anti-VP2 antibodies resulted in the suppression of erythrophagocytosis, we investigated whether or not VP1u is the target for opsonizing antibodies. The VP1u region occupies an internal position in the native particles, and becomes exposed by mild heat (Ros et al., 2006) or after B19 binding to its receptor on RBCs (Bönsch et al., 2008). Parvovirus B19 particles at 2×10^8 DNA copies/ml, were heated or not at 60 °C for 3 min to allow maximal exposure of VP1u at the virion surface (Ros et al., 2006), slowly cooled to 37 °C, and preincubated further at 37 °C for 30 min with 50 μg/ml of IgG samples isolated from patients with past B19 infection. The ability of IgG samples to opsonize RBC-bound B19 particles was then tested using the erythrophagocytosis assay. The results showed maximal suppression of erythrophagocytosis with IgG samples preincubated with heated B19 particles, whereas 10–20% suppression of erythrophagocytosis was observed with IgG samples preincubated with unheated B19 particles (Fig. 5C).

Discussion

During the acute phase of infection, parvovirus B19 viremia usually reaches a peak on days 7 to 9 and is resolved by the development of an

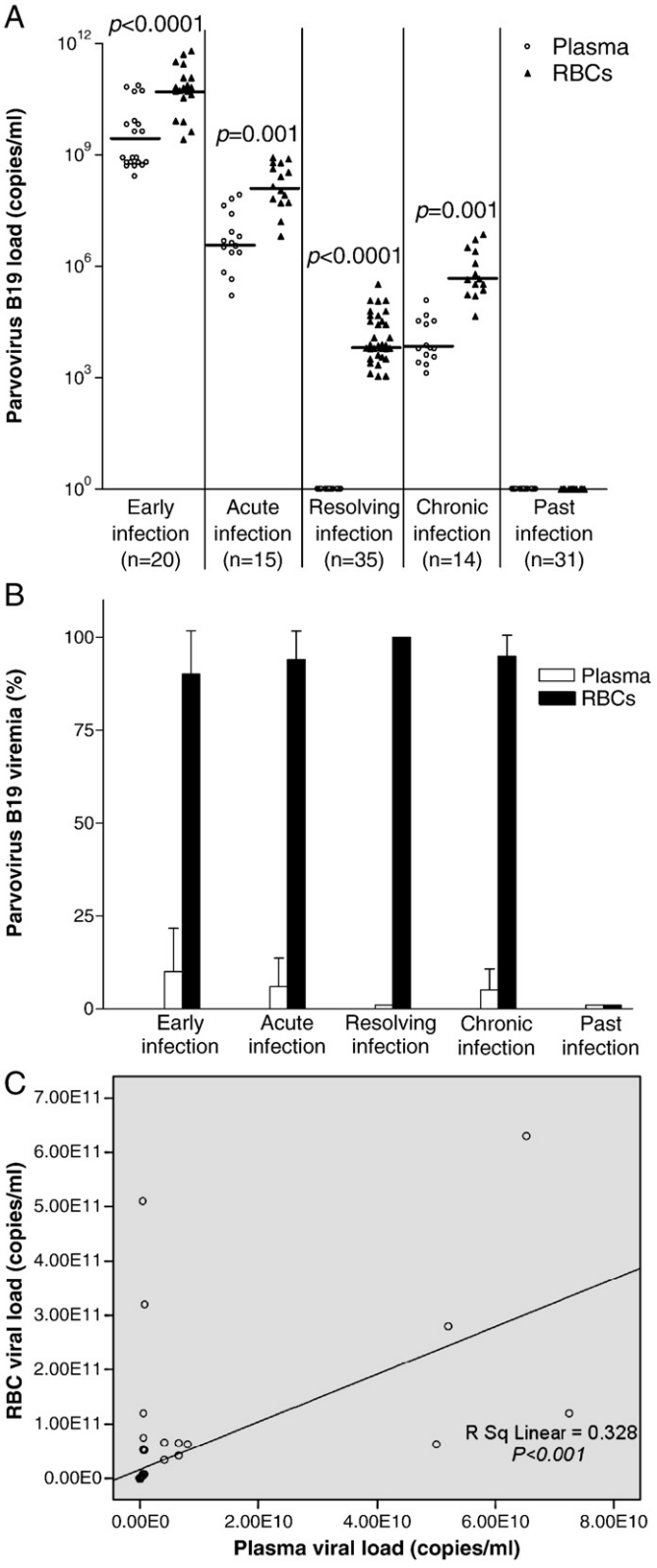

Fig. 1. Parvovirus B19 DNA load in plasma and red blood cell (RBC) fractions. (A) Comparison of parvovirus B19 DNA load obtained by real-time PCR in plasma and RBCs isolated from patients with parvovirus B19 infection at different stages of infection. Horizontal bars represent median values. The two-tailed *p*-values were determined by the Mann–Whitney *U*-test. (B) Percentage of B19 viremia in plasma and RBC fractions from patients segregated according to the stage of B19 infection. Results are shown as means ± standard deviation. (C) Spearman correlation between plasma-associated B19 viral load and RBC-associated B19 viral load.

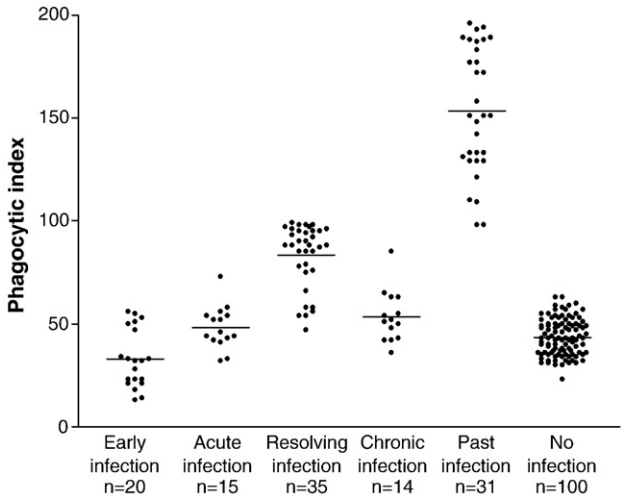

Fig. 2. Evaluation of the ability of plasma from patients at different stages of parvovirus B19 infection to opsonize red blood cells isolated from patients with early B19 infection. Red blood cells containing 10^4 B19 DNA copies/cell, were preincubated for 30 min at 37 °C with decomplemented and adsorbed plasma from control subjects or patients, and then added to monocyte cultures obtained from healthy subjects. Following lysis of noninternalized red blood cells, phagocytosis of red blood cells by monocytes was assessed by a colorimetric assay. Horizontal bars represent median values.

IgM and IgG antibody response, which starts a few days later (Anderson et al., 1985). Parvovirus B19 viremia can also be detected late in some immunocompetent patients despite the development of B19-specific IgG (Cassinotti and Siegl, 2000). The current study showed that most B19 viremia in the blood is associated with RBCs, even in the absence of specific IgM and IgG antibodies which may neutralize plasma B19 virions. The detection of RBC-associated viraemia during convalescence phase indicates that the complete clearance of B19 viremia may take longer time than expected. Human RBCs, which express high levels of B19 receptor (Fletcher et al., 1979), bind B19 virus but do not allow viral entry (Weigel-Kelley et al., 2003). The role of RBCs in the dissemination or clearance of B19 virions is unknown. The erythrophagocytosis assay, described here, has previously been used to assess the immune response to malaria infection (Groux and Gysin, 1990, Mota et al., 1998) and HIV infection (Chan et al., 2001, Kedzierska et al., 2001, Azzam et al., 2006). It was also used to investigate the role of commercial immunoglobulins in the inhibition of autoantibody-mediated erythrophagocytosis (Léonard et al., 2006). Using the erythrophagocytosis assay, we showed, for the first time, that in addition to the hemagglutination properties of B19 particles (Brown and Cohen, 1992), B19 virus may cause erythrophagocytosis mediated by specific opsonizing antibodies. This opsonization was independent of complement system, since all plasma were inactivated by heat treatment, and since opsonization activity was suppressed when IgG were depleted from plasma by affinity chromatography. Furthermore, the opsonization of RBC-bound B19 particles was suppressed in the presence of anti-FcγRII and anti-FcγRIII antibodies, which bind IgG in the form of immune complexes (Gessner et al., 1998).

Parvovirus B19 VP1 and VP2 proteins originate from overlapping reading frames, and are identical except for 227 residues on the amino terminus of VP1, which form the VP1u region. The detection of anti-VP1 antibodies, but not anti-VP2 antibodies, in the opsonizing IgG, by western blot, strongly suggests a role for anti-VP1 antibodies in the opsonization of RBC-bound B19 particles. However, the results of western blot assay cannot exclude the presence of antibodies raised against undenatured VP2 in clinical specimen from patients with past B19 infection, since the immune response to B19 is characterized by a gradual loss of antibodies directed against linear epitopes of VP2 (Söderlund et al., 1995; Franssila et al., 1996). The absence of

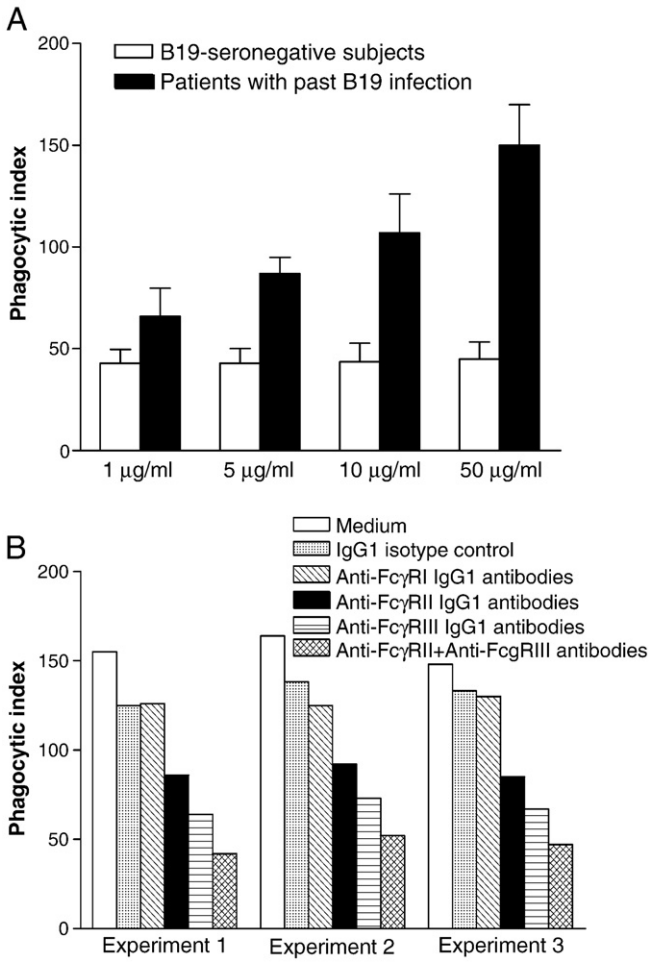

Fig. 3. Opsonization of red blood cells from patients with B19 infection is mediated by specific IgG. (A) IgG eluted from plasma by affinity chromatography, were preincubated at different concentrations with red blood cells containing 10^4 B19 DNA copies/cell, and then added to monocytes from healthy subjects to assess their opsonization activity using the erythrophagocytosis assay. Results are shown as means ± standard deviation of five separate measurements. (B) Plasma IgG (50 µg/ml) from patients with past B19 infection were preincubated with red blood cells containing 10^4 B19 DNA copies/cell, and then added to monocytes preincubated with 0.01 µg/µl of monoclonal IgG1 antibodies directed against FcγRI, FcγRII and FcγRIII. Mouse IgG1 was used as isotypic control in each experiment. Phagocytosis of red blood cells by monocytes was assessed by a colorimetric assay.

suppression of opsonization activity following depletion of IgG samples directed against recombinant VP2 protein expressed in baculovirus system (Brown et al., 1990a, 1990b), suggests that anti-VP2 antibodies do not exert opsonization activity against RBC-bound B19 particles. The detection of anti-VP1 antibodies in the opsonizing IgG depleted of anti-VP2 antibodies, and the suppression of erythrophagocytic activity after depletion of IgG samples on insect cells expressing recombinant VP1 protein (Brown et al., 1990a, 1990b), suggest that VP1u is the potential target for opsonizing antibodies.

VP1u region has been shown to be steadily exposed on the surface of RBCs preincubated with B19 particles (Bönsch et al., 2008), which explains the high opsonization activity obtained after incubation of RBC-bound B19 particles with IgG samples. In free B19 particles, the most N-terminal part of VP1u which harbors strong neutralizing epitopes, and the PLA_2-like motif at the C-terminal region of VP1u, occupy an internal position in the capsid (Ros et al., 2006, Bönsch et al., 2008). Heating B19 particles at 60 °C has been shown to result in maximal exposure of VP1u at the surface of B19 particles (Ros et al., 2006). Our results showed a significant depletion of opsonizing antibodies after preincubation of IgG samples with heated B19 particles, suggesting that the N-terminal part and/or C-terminal part of VP1u region is involved in the opsonization of RBC-bound B19. The partial depletion of opsonizing antibodies obtained after preincubation of IgG samples with unheated B19 particles, may be explained by specific binding to a region of VP1u between the N- and C-terminal, which is exposed on the surface of unheated B19 particles. These observations are in line with a previous report showing that B19 virions in human serum could be immunoprecipitated by using rabbit antisera raised against the entire VP1u (Rosenfeld et al., 1992). However, our results cannot exclude the presence of another opsonizing antigen than VP1u, which needs to be identified.

The impaired opsonization activity of acute plasma specimen containing anti-VP1 and anti-VP2 antibodies, and the enhanced opsonization activity of plasma from past B19 infection, suggest a slow development of anti-VP1u opsonizing antibodies. Previous reports have shown that antibody specificity to the VP2 protein predominates in early B19 infection, whereas the VP1u antibody specificity predominates late during convalescence phase (Kurtzman et al., 1989a). Furthermore, in immunocompromised patients, persistent parvovirus B19 infections may occur despite the presence of circulating B19 antibody (Schleuning et al., 1999; Moreux et al., 2002; Fattet et al., 2004). Similarly, the current study showed that, in spite of having specific anti-B19 VP1 and VP2 antibodies, plasma from patients with persistent B19 infection did not mediate high opsonization activity compared to plasma from patients with past B19 infection. Thus, only antibodies obtained from immune individuals were able to

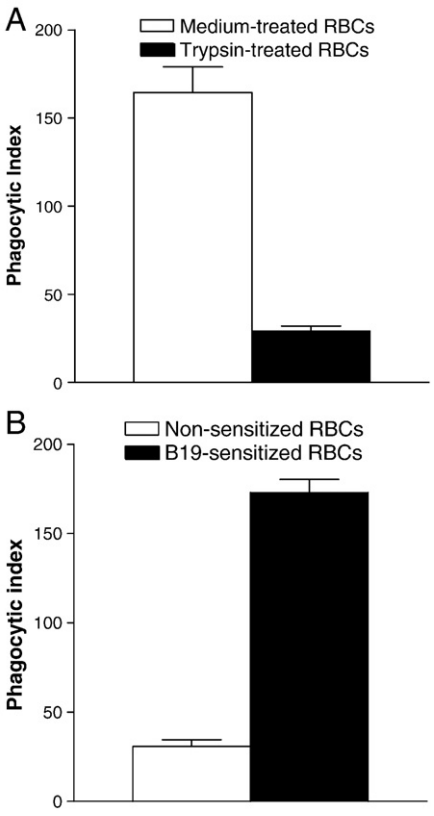

Fig. 4. Parvovirus B19 particles bound to red blood cells are recognized by specific opsonizing antibodies. (A) Red blood cells containing 10^4 B19 DNA copies/cell from patients with early B19 infection, were treated or not with trypsin/EDTA, prior to their incubation with 50 µg/ml of IgG from patients with past B19 infection. Opsonization of red blood cells was then assessed on monocyte cultures using the erythrophagocytosis assay. (B) Red blood cells from healthy subjects were sensitized with 2×10^8 B19 copies/ml or with culture media, and then incubated, after removal of unbound B19 particles, with 50 µg/ml of IgG samples from patients with past B19 infection. Sensitized and nonsensitized RBCs were then added to monocyte-containing wells. Phagocytosis of red blood cells by monocytes was assessed by a colorimetric assay. Results are shown as means ± standard deviation of five separate measurements.

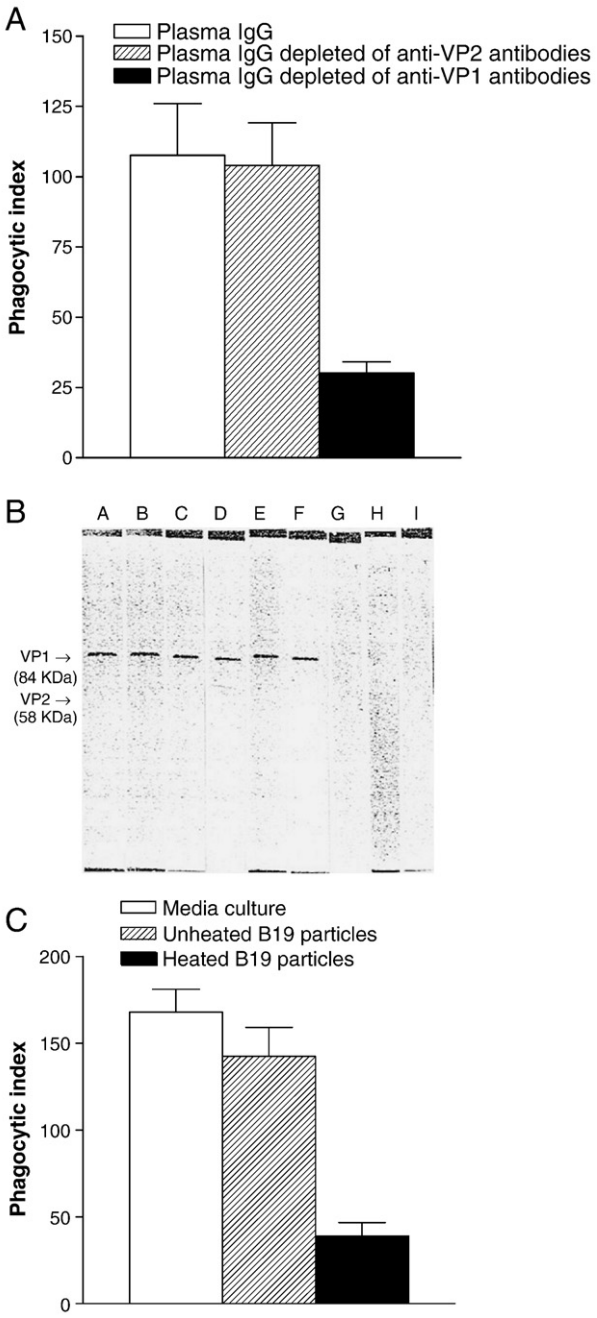

Fig. 5. Parvovirus B19 VP1u region is a potential target for opsonizing antibodies. (A) Antibodies against B19 VP1 or VP2 antigen were depleted from opsonizing IgG samples, by adsorption on insect cells expressing recombinant VP1 or VP2 antigen, respectively. The ability of depleted IgG samples to opsonize RBCs from patients with early B19 infection was assessed on monocyte cultures using the erythrophagocytosis assay. (B) Western blot analysis showing the specificity of opsonizing IgG purified from 3 patients with past B19 infection, before depletion (lanes A–C), after depletion on insect cells expressing recombinant VP2 protein (lanes D–F), and after depletion on insect cells expressing recombinant VP1 protein (lanes G–I). (C) B19 particles at 2×10^8 DNA copies/ml, were heated or not to allow maximal exposure of VP1u at the surface of B19 particles, and then preincubated with 50 μg/ml of IgG samples isolated from patients with past B19 infection. The ability of B19- or medium-treated IgG samples to opsonize RBC-bound B19 particles, was then assessed on monocyte cultures using the erythrophagocytosis assay. Results are shown as means ± standard deviation of five separate measurements.

mediate high opsonization activity, which suggests a protective role for opsonizing antibodies against recurrent B19 infection. The decreased opsonization activity of plasma from persistent B19 infection may be due to the absence of development of enough amounts of specific antibodies directed against VP1u region. These observations are in agreement with previous reports showing a poor immune response against VP1u in persistent B19 infections (Kurtzman et al., 1989a). It is noteworthy that immunoglobulin preparations have been used to treat persistent (Kurtzman et al., 1989b) and acute B19 infection (Herzog-Tzarfati et al., 2006). They contain anti-B19 antibodies which showed specificities to both VP1 and VP2 capsid proteins (Kurtzman et al., 1989a). It would therefore be of considerable interest to determine the amount of opsonizing antibodies in the commercial immunoglobulins, and therefore to be able to predict which immunoglobulin preparation can be of therapeutic value. Furthermore, opsonizing antibodies have been shown to correlate with their protective capacity in vaccination studies (Kaufman et al., 1986; Perraut et al., 1995; Schwenk et al., 2003). The current study suggests that a successful vaccine against parvovirus B19 infection, should be based on the induction of opsonizing antibodies directed against VP1u.

While opsonizing antibodies appear to exert protective role against recurrent B19 infection, they may also have harmful effects. Indeed, antibody opsonization of RBC-bound B19 particles may precipitate severe anemia in patients with hematological disorders who develop recurrent B19 infection. Furthermore, parvovirus B19 has been associated with the development of hemophagocytic syndrome (HPS), characterized by pancytopenia and infiltration of bone marrow and various organs, by histiocytes which are actively involved in hemophagocytosis (Watanabe et al., 1994; Sato et al., 1999; Larroche et al., 2002; Dutta et al., 2005; Yilmaz et al., 2006). Whether antibody opsonization of RBC-bound B19 particles can cause, precipitate or exacerbate HPS is unknown.

In this study, we have shown that B19 viremia was mostly associated with RBCs, and that RBC-bound B19 particles may be opsonized by specific anti-VP1 IgG antibodies isolated from patients with resolving or past B19 infection. Opsonization assay appears as an additional immunological tool to distinguish between immune and non-immune individuals. The unability of patients with persistent B19 infection to clear the virus may be related to a defect in the production of specific opsonizing antibodies directed against VP1u region. The production of opsonizing antibodies in persistent parvovirus B19 infection should be investigated in high number of patients to better understand the development of immunity to B19 infection. Furthermore, the IgG subclass involved in the opsonization activity needs to be identified since a balance between IgG subclasses may determine whether or not a plasma specimen can opsonize RBC-bound B19 particles.

Materials and methods

Collection of clinical samples

The clinical specimens for this study were received during the period between September 2006 and October 2008. Venous blood specimens were collected in 5 ml EDTA tubes from patients suffering from fever and rash followed or not with arthralgia (97 children, 41 adults), or from chronic hemolytic anemia (92 children, 70 adults). They were enrolled from outpatient departments of Farwaniya, Mubarak Al-Kabeer, and Al-Sabah hospitals. Blood specimens were also collected from 40 kidney transplant recipients with transient aplastic crisis (TAC). They were recruited from Hamad El-Essa Organ Transplant Center (OTC), and were receiving immunosuppressive drugs at the time of the study. Blood samples tested positive for cytomegalovirus (CMV) DNA, Epstein–Barr virus (EBV) IgM, human herpes virus type 6 (HHV-6) DNA, rubella virus IgM, measles virus IgM, adenoviral DNA, or enteroviral RNA were excluded from this study. The control group included 100 control subjects tested negative for B19 DNA, IgM and IgG. They had no fever or any disease known to be associated with viral infection. The ethical permission on this

research study was granted by the Ethical Decision Committee of the Research Administration, Faculty of Medicine, Kuwait University.

Virus and antibodies

Serum samples containing high titers of parvovirus B19 DNA were obtained in our laboratory in the course of routine virological screening. They were isolated from B19-infected patients during the early viremic phase, and tested negative for B19-specific IgM and IgG antibodies. Ten ml of B19-containing serum samples was diluted 1:2 in phosphate-buffered saline (PBS, pH 7.4), and preincubated overnight at 4 °C with protein L and G agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) to remove immunoglobulins and immunocomplexes. After centrifugation at 1000 ×g for 5 min, the B19-containing supernatant was clarified by centrifugation on a Macrosep® 300 kDa Omega membrane (Pall Life Sciences, Ann Arbor, MI), and resuspended in 1 ml PBS, pH 7.4. Parvovirus B19 DNA load was obtained by quantitative real-time PCR as described below. Monoclonal IgG1 neutralizing anti-human FcγRI (CD64) clone 10.1, monoclonal IgG1 neutralizing anti-human FcγRII (CD32) clone AT10, monoclonal IgG1 neutralizing anti-human FcχRIII (CD16) clone DJ130c, and mouse IgG1 negative control were purchased from AbD Serotec (MorphoSys UK Ltd, Oxford, UK).

Blood cell separation

Plasma and mononuclear cells (MNCs) were separated from blood samples by centrifugation on a Ficoll-Paque™ Plus density gradient (GE HealthCare Bio-Sciences, Uppsala, Sweden). Monocytes were negatively isolated from MNCs of control subjects by depletion of other blood cells using specific immunomagnetic beads (Dynal Monocyte Negative Isolation Kit, Invitrogen Dynal AS, Oslo, Norway). They were resuspended in serum-free RPMI 1640 medium supplemented with 2 mM L-glutamine, seeded at 5×10^4 cells per well into 96-well flat-bottom tissue culture plates, and allowed to adhere for 60 min at 37 °C before being used in the erythrophagocytosis assay. Red blood cells (RBCs) from patients and controls were collected and washed three times with PBS, pH 6.7, and resuspended at a concentration of 5×10^6 RBCs/ml in an isotonic salt solution (Alsever's solution, Sigma Aldrich, St. Louis, MO). Red blood cell suspensions were kept stored at 4 °C until further use (up to 2 weeks).

Detection of B19-specific IgM and IgG

The seroprevalence of B19 infection in patients and control subjects was determined by detecting plasma B19-specific IgM and IgG using immunofluorescence assays (IFA) from PanBio (PanBio Inc, Baltimore, Maryland) and Biotrin (Biotrin International, Dublin, Ireland) as described by the manufacturers' instructions. The PanBio tests were based on the detection of antibodies directed against recombinant B19 VP2 antigen, and the Biotrin test on the detection of antibodies directed against recombinant B19 VP1 antigen. A plasma sample was considered B19 antibody positive if it was reactive on both tests.

Detection and quantification of B19 DNA

Total DNA was extracted from 140 µl of plasma samples and from 5×10^5 RBCs using Qiamp® DNA Blood Mini kit (QIAGEN Inc., California, USA), as described by the manufacturer's instructions. The B19 DNA was amplified by nested PCR using previously described primers and conditions (Candotti et al., 2004). The PCR reaction was carried out with a Perkin-Elmer Applied Biosystems Gene-Amp 9700 thermocycler. Parvovirus B19 DNA cloned into pGEM®-T Easy vector (Promega Corporation, Madison, WI), was used as positive control. The B19 viral load was determined on the LightCycler® 2.0 Real-Time PCR system by using the Parvovirus B19 Quantification Kit (Roche Molecular Biochemicals). As noted in the manufacturer's instructions, the parvovirus variants A6 and V9 are not detected with this assay. The linear range of the assay was from 10^2 to 10^6 copies per reaction.

Erythrophagocytosis assay

One ml of plasma from control subjects or patients, was heated at 56 °C for 30 min to inactivate complement components. To prevent unspecific binding to RBCs, 1 ml of decomplemented plasma was adsorbed at room temperature for 30 min, with 0.5 ml of normal human RBC pool prepared by mixing equal volume of RBCs from B19-seronegative control subjects with different blood group. RBC suspensions obtained from patients with early B19 infection at preseroconversion phase, were washed three times with PBS, and then resuspended at 5×10^6 RBCs/ml in PBS pH 6.7, containing 3% bovine serum albumin (BSA). B19 viral load in RBC suspension was measured by real-time PCR, and the number of RBCs used for erythrophagocytosis assay was adjusted to contain 10^4 B19 DNA copies/RBC. In some experiments, RBC suspensions were treated with trypsin-EDTA (0.05% trypsin plus 0.02% EDTA) for 5 min at 37 °C to remove B19 particles from the surface of RBCs as described previously (Weigel-Kelley et al., 2003). One hundred µl of RBC suspension was preincubated with 100 µl of decomplemented and adsorbed plasma for 30 min at 37 °C in a shaking waterbath. RBCs were then washed 3 times with 5 ml PBS, resuspended in 100 µl of serum-free RPMI 1640 medium supplemented with 2 mM L-glutamine and 2 µM β-mercaptoethanol, and then added to the monocyte-containing wells. Phagocytosis of RBCs was terminated after 1 h incubation at 37 °C and 5% CO_2, by placing the plate on ice, and the wells were washed thoroughly with cold PBS to remove unbound RBC. Adhered but non-phagocytosed RBCs were lysed with 0.2% NaCl solution for 3 min, and removed by washing three times with prewarmed RPMI 1640 medium. Monocytes were then lysed with 6 mol/L urea solution containing 0.2 mol/L Tris–HCl buffer pH 7.4. The number of internalized RBCs was determined using previously described colorimetric assay (Gebran et al., 1992). In brief, 10 mg/ml of 2,7-diaminofluorene (Sigma Aldrich) was added, and the absorbance at 620 nm, which is proportional to the amount of fluorene blue released in the presence of the pseudoperoxidase activity of hemoglobin, was measured. The number of phagocytosed RBCs was determined by extrapolation from a standard curve generated using known amounts of RBCs (ranging from 5×10^3 to 5×10^6). The number of RBCs phagocytosed per 100 monocytes, usually referred to as phagocytic index, was obtained by dividing the number of phagocytosed RBCs by the number of monocytes in the well (5×10^4 cells), and multiplied by 100.

IgG affinity purification

Plasma samples (2 ml) collected from patients with past B19 infection or from B19-seronegative subjects, were decomplemented and adsorbed with 1 ml of normal human RBC pool as described above. They were then diluted twofold with PBS at pH 7.0, filtered (0.45 µm), and passed at a flow rate of 0.1 ml/min over a 1.6 cm×20 cm column of Protein G-Sepharose™ Fast Flow (GE Healthcare) equilibrated with PBS buffer. The column was then washed extensively with the same buffer until the absorbance at 280 nm of the effluent was zero. Elution of IgG was performed at a flow rate of 1 ml/min with elution buffer containing 0.1 M glycine HCl pH 2.5. To preserve the activity of acid-labile IgG, the eluted fractions were neutralized by adding 100 µl of 1 M Tris–HCl, pH 7.4 to each milliliter of eluted fluid. Eluted IgG was concentrated using Biomax-30 membrane (Millipore Corp., Bedford, MA) according to the manufacturer's instructions. IgG concentrations were estimated using an assumed extinction coefficient of 1.43 for 1 mg/ml solution at 280 nm.

In vitro sensitization of RBCs with parvovirus B19

One hundred μl of RBCs from healthy subjects was preincubated for 2 h at 4 °C with 100 μl of B19 virus at 2×10^8 DNA copies/ml. After excessive washing with PBS to remove unbound B19 particles, sensitized RBCs were resuspended in 100 μl of PBS, and a fraction was used to quantify RBC-associated B19 by real-time PCR. Sensitized RBCs with a B19 viral load of 10^4 copies/RBC were then used in the erythrophagocytosis assay described above.

Depletion of anti-parvovirus B19 VP1 and VP2 antibodies

Fifty μl of purified plasma IgG (50 μg/ml) from patients with past B19 infection, was added either to the well slides coated with insect cells expressing parvovirus B19 recombinant VP1 antigen (Biotrin International), or to the well slides coated with insect cells expressing parvovirus B19 recombinant VP2 antigen (PanBio® Inc). Following 30 min incubation at 37 °C in a moist chamber, IgG samples were harvested from the well slides and tested for their ability to opsonize RBC-bound B19 using the erythrophagocytosis assay.

Western blot analysis

Ten μl of parvovirus B19 suspension, prepared as described above, was added to 20 μl of Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA) containing 2% β-mercaptoethanol, and heated at 100 °C for 5 min, before being loaded into 12% SDS-polyacrylamide gel. One μl of the Precision Plus Protein™ Standards marker (Bio-Rad Laboratories) was dissociated in the same way, and applied to the left side of the gel. Following electrophoresis using the Mini-Protean® Tetra Cell (Bio-Rad Laboratories), the separated proteins were electroblotted onto a nitrocellulose membrane (Bio-Rad Laboratories) using the Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad Laboratories), and then blocked with Tris-buffered saline (TBS) buffer pH 7.5, containing 3% gelatin, at room temperature for 1 h. Following washing with TBS buffer containing 0.05% (v/v) Tween-20, the membrane was cut into strips, and each strip was incubated, for 1 h at room temperature, with opsonizing IgG sample diluted at 1:50 in TBS buffer containing 1% gelatin and 0.05% (v/v) Tween-20. The reactivity of human IgG with parvoviral proteins was detected using the Immun-Blot goat anti-human IgG (H+L) alkaline phosphatase assay kit (Bio-Rad Laboratories) according to the manufacturer's instructions.

Statistical analysis

Mann–Whitney *U*-test was used to compare median values. Spearman's rank test was used to investigate the correlation between plasma-associated viral load and RBC-associated viral load. Probability levels less than 0.05 by the two-tailed test were considered significant. All statistical analyses were performed using SPSS 15.0 software for Windows (SPSS, Inc., Chicago, IL).

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